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13. ABSTRACT (Maximum 200 Words)

The purpose of this research is to develop a new technique of using carbon nanotubes for breast cancer detection, diagnosis and killing of cancer cells. This can be achieved by fabrication of single wall carbon nanotubes, separation of carbon nanotubes, functionalization of antibodies on carbon nanotubes and studying the interaction of carbon nanotubes on cancer cells. This research investigated the effects of functionalization of carbon nanotubes (CNTs) with a primary monoclonal mouse immunoglobin (IgG) specific to the cell-surface receptors of breast cancer cells. Co-localization for CNTs in combination with the primary antibody conjugated to the secondary was determined to be 90%. Specific functionalization of antibodies on carbon nanotubes surfaces were achieved by using an amine terminated PEG molecule that specifically attaches to the primary antibody specific to the cell surface receptors of the breast cancer cell. Preliminary studies on the electrical measurements of the primary mouse IgG incubated with CNTs show a decrease in conductance compared to that of bare CNT field effect transistors (CNTFETs). This observed change in conductance is being currently used as the basis to develop a biosensor for single cell cancer detection systems.

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Table of Contents

I. Introduction

In 2004, invasive breast cancer will attack approximately 190,000 US women, and this malignancy will take the lives of approximately 41,000 patients. Breast cancer is the leading cause of cancer in US women (excluding skin cancers). Diagnostic systems that can detect cancer at an early stage can be a priceless gift to patients suffering from this disease. With progress in the area of nanotechnology, it is now possible to synthesize nanotubes and nanowires approaching the size of proteins or DNA or other functional biological molecules (1.5 nm to 10 nm). Keeping these rapid advances in mind, this research presents a revolutionary approach of developing a nano-biohybrid system where, nanowires and nanotubes are used as probes for detecting and killing cancer cells by assembling specific anti-oncogene antibodies on nanowire/nanotube surfaces for detecting over expressed cell surface receptors called Her2 in cancer cells. The hypothesis used is that assembling antibodies on the nanowire/nanotube surfaces will change the surface electronic and optical properties of the material that can be detected using tunneling, confocal microscopic and field effect modulation techniques. This hypothesis will work as most of the atoms in the nanotubes and nanowires are surface atoms. Hence modulation of surface electronic charge density by functionalizing antibodies can change the electronic and optical properties dramatically, which can be used to develop highly sensitive sensors for detecting breast cancer cells approaching the limits of being able to detect single cancer cells. This would be a significant step in the area of biomedical nanotechnology and could revolutionize cancer diagnostic systems as we will be able to nanoelectronically detect a single cancer cell. Further, the success of this technology can result in cancer diagnostic systems for various other types of cancer based on similar principles that can be used in a clinical set up for early detection of cancer.

II. Body

II. A. Carbon Nanotube Synthesis:

In this research, the first task was to fabricate nanoparticles, nanowires and carbon nanotubes. To this, we started fabricating carbon nanotubes using a methane based chemical vapor deposition approach. We found that nanotubes were much more suited for our research for coating antibodies due to the inert nature of carbon nanotubes and their biological compatibility. Further, carbon nanotubes show interesting metallic and semiconducting properties which is the focus of this research.

Carbon nanotubes are first grown in-house using methane based thermo chemical vapor deposition technique at 900 °C at atmospheric pressure using 10 nm iron nanoparticles as catalyst metal. The growth is set in the reaction rate limited regime with high temperatures facilitating high kinetic energy of the gas molecules and with low supply of carbon, allowing the formation of single wall carbon nanotubes. The grown nanotubes are purified by first heating in dry air at 400 °C for removing the soot and an acid reflux (3 M HCL for 10 hrs) to remove the catalyst particles. Following growth, the nanotubes were characterized using scanning electron and transmission electron microscopes. Figure 1(a), (b) & (c) is the SEM and TEM image of the nanotube grown using this approach about 1.5 nm in diameter and 1 micron in length. The PIs group at University of Delaware has grown nanotubes about 0.9 nm to 10 nm in diameter and 1 μm in length.

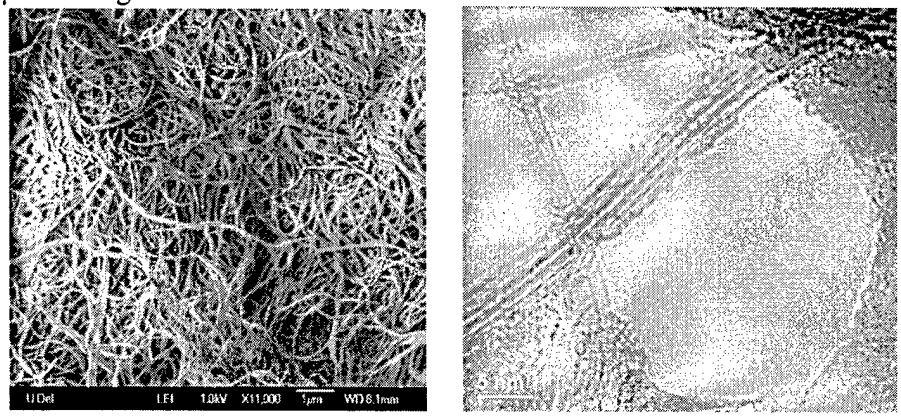

Figure 1. (a) Scanning electron microscope image of bundles of single wall carbon nanotubes, (b) Transmission electron microscopic image of single wall carbon nanotubes of 1.5-1.6 nm in diameter.

II.B Characterization of Nanotubes for their Quantum Electronic Properties:

In this task the quantum conductance of the nanotubes and nanowires were measured and the differential conductance as a function of voltage was measured using nanoprobes. We used atomic force microscope tips and connected it with the regular probes tips to measure the conductance which is the most direct way of measuring conductance in carbon nanotubes directly. Atomic force microscope tips have a tip radius of 17 nm which is quite close to carbon nanotubes (1.5 nm to 10 nm). Using these tips the nanotube conductance can be measured

directly using probe stations that is conducted to a semiconductor parameter analyzer. The probe tips were initially welded to the base of the AFM tip and the tip was positioned on the substrate consisting of separated carbon nanotubes. Figure 3 is the micrograph of the differential conductance measured for carbon nanotubes and platinum nanowires. The gap is the conductance across V=0 gives the energy or conductance gap. The step like behavior is associated with the non-linear current voltage characteristics of carbon nanotubes. These nanotubes are semiconducting in nature and this characterization allows for measuring the base-line conductance of carbon nanotubes.

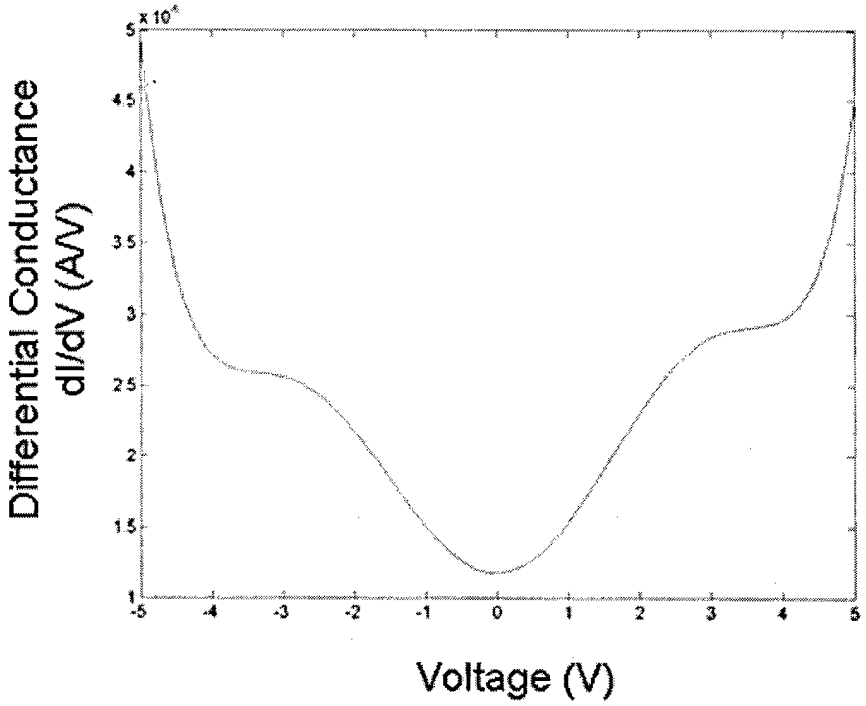

Figure 2. Differential conductance as a function of voltage for carbon nanotubes between -5 to +5 V. Note that the gap at V=0 gives the conductance or energy gap.

II C. Antibody Functionalization of Carbon Nanotubes:

In order for nanotubes and nanowires to be used for biomolecular recognition, it is important to first separate the nanotubes and nanowires, measure the conductance, functionalize antibodies on nanotube and nanowire surfaces and measure the conductance of the antibody coated nanotubes and nanowires. Confocal microscopy was used to characterize the functionalization of nanotubes and nanowires with antibodies. This is also the first time to visualize carbon nanotubes directly using confocal microscopes as they are very small to be observed. For observing in confocal microscopes, the nanotubes have to be first separated and labeled using flourophores. The first step was the separation of nanotube bundles into individual nanotubes.

II.C.1. Separation of Nanotube Bundles into Individual Nanotubes:

Most applications employing the unique electronic, thermal, optical and mechanical properties of individual nanotubes will require large scale manipulation of stable suspensions at high weight fraction. Nanotube solubilization provides access to solution-phase separation methodologies, facilitates chemical derivatization and controlled dispersion which is critical for antibody functionalization.

For controlled dispersion and solubilization, the CNTs were prepared in a solution of distilled water at a density of approximately 0.33mg/ml and solubilized in sodium dodecylbenzene sulphonate (ICN Biomedicals Inc.), a surfactant that ensured their separation in aqueous environment at a ratio of 1:20 of CNT: surfactant by weight [1]. The entire mixture was gently agitated for 24 hours in a sonicator that resulted in non-specific adsorption of surfactant on the sidewalls of the carbon nanotube and separation of the nanotube bundles into individual nanotubes. To view in an SEM, a drop of the nanotube-surfactant mixture was placed on a silicon wafer and the sample was viewed after evaporation of the solvents. Figure 3 (a) is the SEM image of carbon nanotube bundles, and Figure 3 (b) is the SEM image of separated nanotubes. These results are in very good agreement with the results reported previously in the literature. We have found that NaDDBS is the most effective for our nanotubes grown in our laboratory. The superior dispersing capability of NaDDBS can be explained in terms of graphite-surfactant interactions, alkyl chain length, head group size and charge as pertains to the molecules that lie along the surface, parallel to the central tube axis. Figure 3 (c) is the schematic illustration of how a surfactant may adsorb on to the nanotubes. It is because of the π-like stacking of the benzene rings onto the surface of the graphite that causes the surfactant to bind to the surface of the nanotube. Previous studies have reported the lack of adsorption of other surfactant molecules such as SDS on nanotube surfaces [2]. This is attributed due to the absence of benzene rings on the surfactant molecules and our study shows the effectiveness of using NADDBS as surfactant for this study.

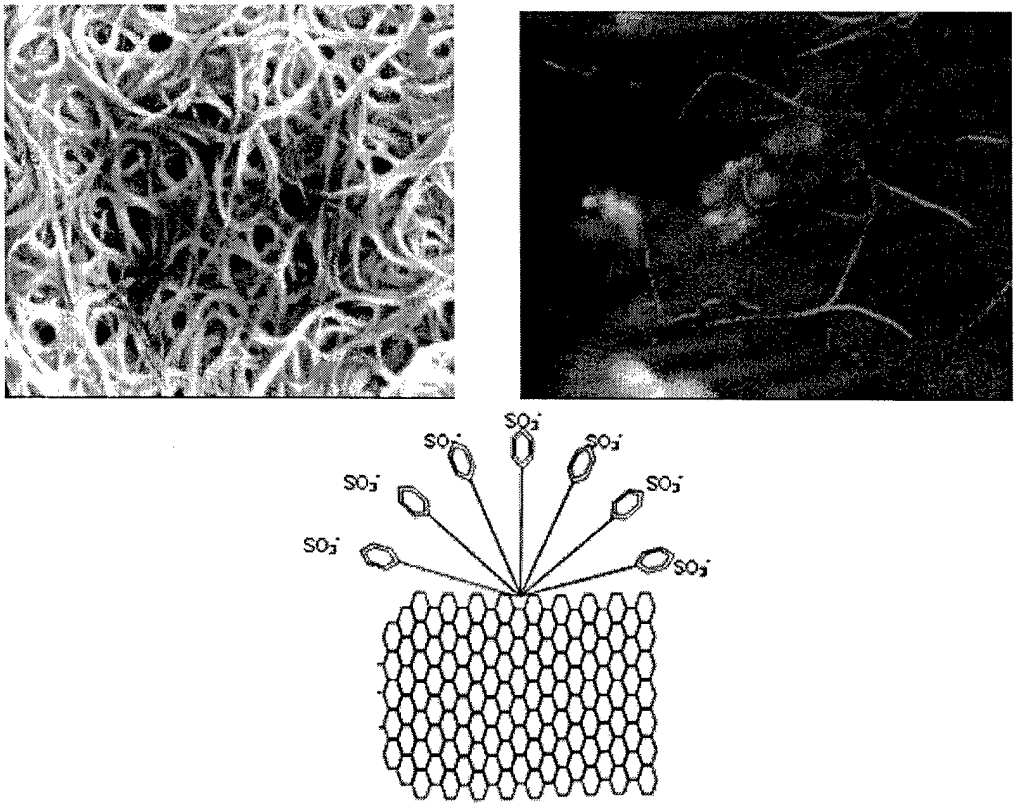

Figure 3 (a) Carbon nanotube in bundles before use of surfactant (b) nanotube bundles separated into individual tubes (c) schematic illustration of how surfactant may adsorb onto the nanotube surface to separate the nanotubes.

II.C. 2. Visualization of Individual Carbon Nanotubes in Confocal Microscope using Conventional Flourophores:

Labeling CNTs with conventional flourophores places several advantages for studying the interaction of biological molecules on carbon nanotubes. First, it allows the visualization of smaller carbon nanotubes approaching individual nanotubes using confocal microscopy with out aid from electron microscopic techniques. It doesn't damage the nanotube lattice thereby preserving the electronic transport properties of the nanotubes. When antibodies are functionalized on the nanotube, the resultant change in the electronic properties of the nanotube stems from the antibodies as the flourophores does not change the Sp^2 bonded grapheme sidewall. Further, nanotubes coated with flourophores can also be used as contrast agents to visualize internal parts of the cellular structure and even may enable high contrast imaging for breast cancer imaging and vaccine delivery applications using carbon nanotubes. For successful imaging and for higher contrast images, it is essential that the nanotubes are well separated and labeled using flourophores. In the recent past, the interaction of carbon nanotubes with different types of flourophores has been successfully investigated [3]. It was shown that nanotubes could

be labeled successfully using DiIC16 and DiOC6. It was found that DiOC2, DiOC5, BODIPY or hydrazide did not produce any successful labeling of carbon nanotubes.

In our preliminary experimentation with flourophores, we have integrated our nanotube fabrication, purification and separation technology using surfactant solutions, with labeling using DiOC6, thereby preserving the sidewalls of the nanotube for tagging biologically active molecules such as antibodies. We also don't see any carbonaceous residue as all the nanotubes that we use for this purpose are purified nanotubes.

Single wall carbon nanotubes after fabrication were ultrasonically agitated for upto 8 hours in water at a density of approximately 0.001mg/ml, and labeled with dihexyloxacarbocyanine iodide ($DiOC_6$ – Molecular Probes Inc), by allowing interaction of the two components for 2-3 hours. Following labeling, the surfactant solution was added to make CNT:surfactant of 1:20 by weight as mentioned above and agitated for 6 hours. The resultant samples were imaged using Zeiss LSM 510 Multiphoton Confocal Microscope. Figure 4 (a) is the confocal image of nanotubes treated with DiOC6 with out the use of any surfactants and Figure 4 (b) is the fluorescence image of nanotubes treated with surfactant solution. This shows that well separated CNTs could be easily located in a fluorescence image and is an important step for studying the interactions of biomolecules on nanotubes using confocal microscopy. The smallest nanotube that has been viewed using confocal microscopy is ~10 nm using $DiOC_6$. The addition of surfactant increases the contrast during fluorescent imaging of the carbon nanotubes and reduces background noise due to the separation of the nanotubes. This study also shows that carbon nanotubes could be used as contrast agents in the study of cancer cells in vivo. Nanotubes coated with fluorescent labels or radioactive dyes can be injected into the body and conventional techniques such as Magnetic Resonance Imaging can be used to locate the nanotubes within the tissues. This is very important if nanotubes are to be used as drug delivery vehicles and the present study clearly shows the feasibility of such an approach. However, more studies needs to be done on the in vivo utility of carbon nanotubes as it is not clear whether injecting a nanotube into tissues in mice or human body is safe.

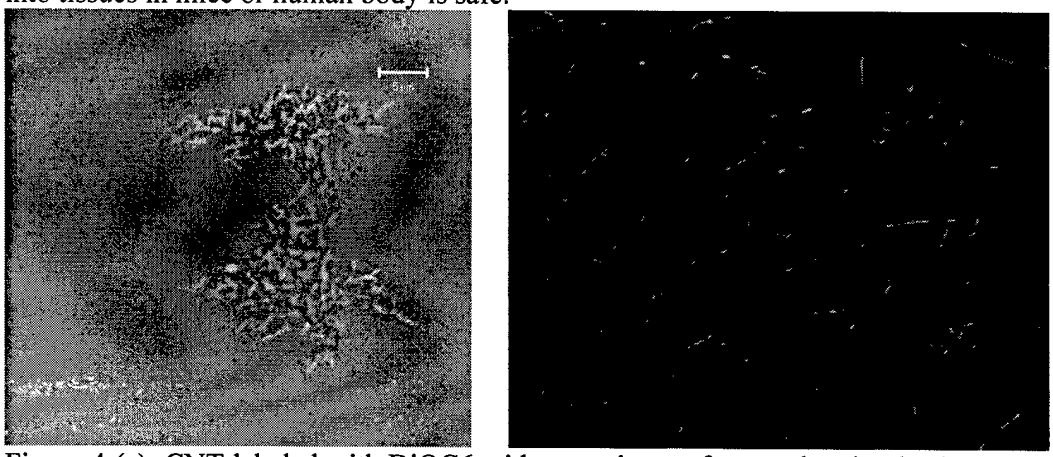

Figure 4 (a): CNT labeled with DiOC6 with out using surfactant showing background noise and no contrast (b) Nanotubes labeled using DiOC6 and separated using NaDDBS surfactant solution showing better contrast and no background noise. Note: both images have 5 micron scale bar.

II. C. 3. Antibody Functionalization of Carbon Nanotubes:

The CNT solution was prepared by agitating the CNTs for 24 hours after adding sodium dodecyl benzene sulfonate, a surfactant, to the solution at a ratio of 1:20 (CNT : surfactant) by weight. The nanotubes were then labeled with dihexyloxacarbocyanine iodide ($DiOC_6$), a dye that fluoresces at 488nm. The $DiOC_6$ was prepared at 2mg/ml in methanol and diluted in distilled water just prior to use with the CNT solution. The dye and nanotube solution were mixed at a 1:1 ratio and allowed to incubate for 1-2 hours. They were then viewed using a Zeiss LSM 510 Multiphoton Confocal Microscope to verify their dispersion.

Two antibodies were used, a secondary polyclonal goat anti-mouse IgG (Molecular Probes Inc.) and primary mouse monoclonal IgG (EMD Biosciences). The secondary antibody is only for labeling purposes to visualize in a confocal microscope. It is the primary monoclonal mouse IgG that will dock with the Her2 cell surface receptors in cell experiments. The antibodies were prepared in Phosphate Buffered Saline (PBS) solution (0.138M NaCl, 0.0027M KCl, pH 7.4), by diluting a 2mg/ml antibody solution with PBS to a ratio of 1:10 (antibody : PBS), just prior to use. The secondary antibody was pre-labeled with Alexa 546 (Molecular Probes Inc), a dye that fluoresces at 543 nm. The CNT solution and secondary antibody solution were then mixed in a micro-centifuge tube and allowed to interact for up to 2 hours. Centrifuging was done to the antibody solution when necessary to eliminate unnecessary fluorescence. The un-labeled primary was first tagged with the fluorescently labeled secondary antibody by allowing them to interact for ~1 hour, and then introduced to the CNT solution in the same way as the secondary.

We have used confocal microscopy to view fluorescently tagged CNTs in a method similar to that of viewing biological proteins such as antibodies to accurately analyze and quantify their interaction [4]. Co-localization is seen to increase with incubation time as evaluated by the change in weighted co-localization coefficient (WCC) [5], which is defined as the ratio of the intensity of co-localized area of a particular channel (color) to the intensity of total area above threshold intensity of that channel (color). The value of WCC was observed to increase from 65% to 88% for the red channel with the increase in incubation time from 5 min to 2 hours between the nanotubes and secondary antibodies as previously reported in our earlier work [6]. This is the first time to show such hifgh degree of functionalization of antibodies on nanotube surfaces. A high degree of co-localization (88%) was seen between CNTs and secondary rabbit anti-goat IgG antibodies upon ~2 hours of incubation in Figure 5(a).

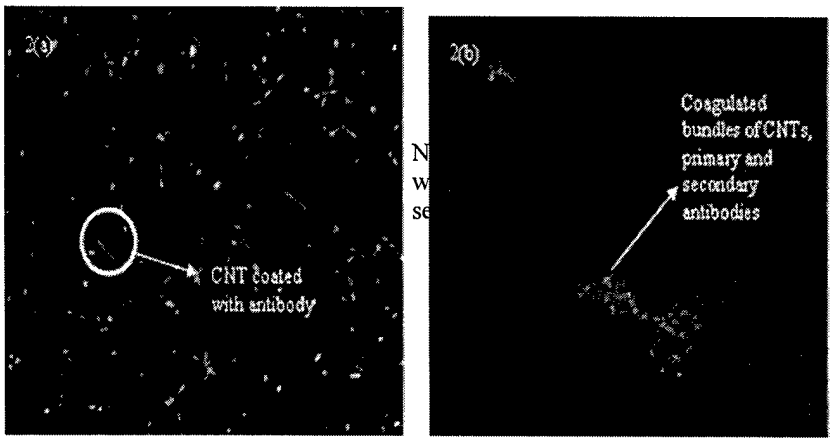

Figure 5: (a) Flourescence image of CNTs functionalized using secondary rabbit anti-goat IgG antibodies upon 2 hours of incubation. The WCC was measured to be 88% showing high degree of co-localization of secondary antibodies on nanotubes; (b) Flourescence image of nanotubes with primary and secondary antibodies

To effectively view and evaluate binding of the primary antibody (monoclonal mouse IgG) to the CNTs, the primary antibodies were conjugated with secondary antibody (polyclonal goat anti mouse IgG). Upon viewing the primary antibody in conjugation with the secondary on the nanotubes, considerable binding was observed (see Figure 5 (b) top corner), although separation of the CNTs was no longer effective (Figure 5(b)). The effect can be attributed to the fact that the binding site is formed due to clustering of the hydrophobic amino acid groups that interact with the CNT surfaces similar to the fullerene interaction on antibodies as referenced in [7, 8]. When the primary and secondary IgG interact, the resultant cohesive forces force the antibodies together and the nanotubes that are bound to them.

To verify the selective attachment of antibodies on carbon nanotubes, the nanotubes were labeled with a green dye using DiOC6 and the antibodies with a red dye. Figure 6 (a) and (b) shows the selective attachment in confocal microscopy analysis. Confocal microscopy image of a green-dye-labeled CNTs and subsequently coated with the red-dye-conjugated antibodies. The green dye (labeling CNTs) in the inner rectangular region is bleached to verify the selective attachment of the antibodies to the CNTs (i.e. CNTs appear in red color due to the antibody binding) and the weighted co-localization coefficient was found to be 88% for antibody functionalization.

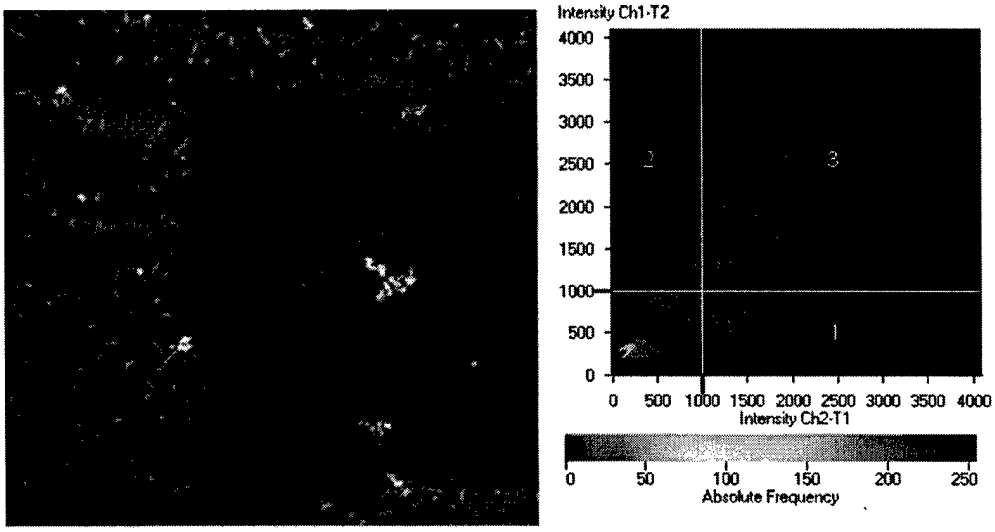

Figure 6: (a) Confocal microscopy image of a green-dye-labeled CNTs and subsequently coated with the red-dye-conjugated antibodies. The green dye (labeling CNTs) in the inner rectangular region is bleached to verify the selective attachment of the antibodies to the CNTs (i.e. CNTs appear in red color due to the antibody binding); (b) WCC found to be 88% for antibody functionalization.

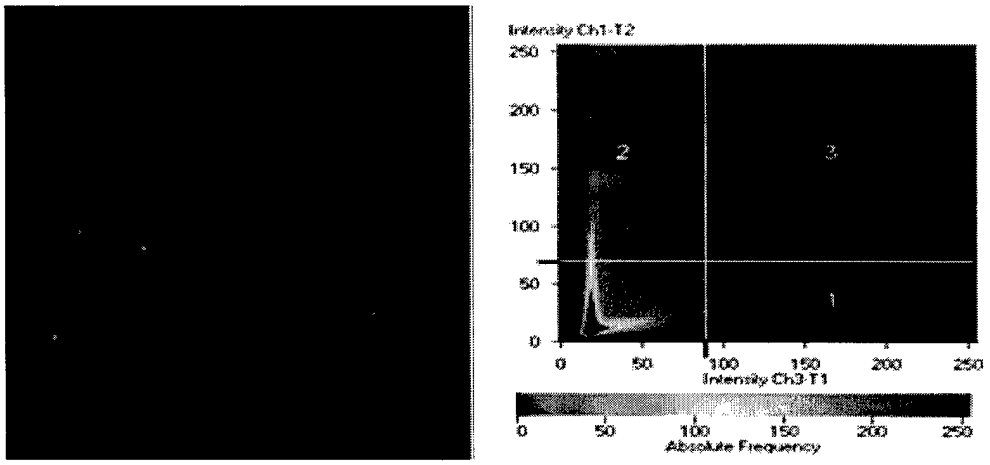

Figure 7: (a) Confocal images of (a) CNTs coated with $DiOC_6$ (green) and incubated with PEG for ~2 hours (b) Graph to mark the threshold intensities of the WCC data; (region 1 represents the red channel, region 2 the green channel and region 3 represents the co-localized area); WCC found to be 0% for the co-localized regions.

To prevent non-specific binding of antibodies to the CNTs, polyethyleneglycol (PEG), a bio-compatible polymer that has been widely used to prevent non-specific binding of proteins to the surfaces of nanostructures in sensing applications, has been used against the secondary IgG. In studies on specific binding of streptavidin on CNT surfaces, the hydrophilic PEG molecules

were used to block the streptavidin molecules from binding to the CNT surfaces [9]. We have performed similar experiments with antibodies and are seen to produce the same effect. Figure 7 (a) is a confocal microscope image of the CNTs (green), coated with PEG and then incubated with the primary IgG combined with secondary IgG (red) for ~2 hours. Upon co-localization analysis, under the influence of PEG, co-localization coefficient (WCC) in Figure 7 (b) is seen to be zero.

One method of measuring the degree of co-localization of objects in confocal dual-color images is calculation of co-localization coefficients. It was reported that the co-localization coefficients can provide quantitative information, when the numbers of the objects in the two components of the image are not equal[18]. In order to measure the degree of co-localization between CNTs (green) and antibodies (red), multiple images were obtained from each sample. Furthermore, the images were taken under the same conditions. The images were then analyzed using the Zeiss LSM 510 software by creating a scattergram, which is interactively linked to data table and the image display, for each image. Threshold intensities in the scattergram are determined such a way to eliminate the background intensities. In fact, the threshold intensities for both channels (red and green) were kept same for all the images. The co-localization data is presented in Table 1.

Samples	CNT co-localization coefficient	Antibody co-localization coefficient
1	0.84	0.86
2	0.69	0.97
3	0.62	0.85
4	0.86	0.92
5	0.89	0.76
6	0.92	0.87

Table 1. Co-localization coefficients for CNTs and antibodies.

II.D. Atomic Force Microscope (AFM) Imaging of Primary Antibodies and Conductance Measurements:

The energy gap between the valence and conduction band is of fundamental importance to the properties of solid. Most of the material's behavior, such as intrinsic conductivity, optical transitions and electronic transistions depend on it. Any change in the gap can alter the material physics and chemistry. The effects such as structural changes, lattice construction, atomic relaxation, surface passivation, surface reconstruction, strain induced changes due to coating, can change the local electronic density of states and the energy gap which is a quantum effect, and can result in dramatic modulation in the conductivity. It is this quantum effect that we tend to take advantage of in sensing cancer cells using nanotube probes. Initial studies on 5 nm silicon nanoparticles have shown that the band gaps can change with particle size (quantum size effect), electronic coupling between particles and cross-linking [10]. Nanotubes have many of their atoms in their surface positions. A surface is a strong perturbation to any lattice, creating many dangling bonds. These unsaturated bonds are energetically unfavorable. The dangling bonds, originally present at the surface of the nanotube can be passivated with their interacting neighbors (antibodies), thereby reducing their energy and in the process changing their localized surface electronic charge density and conductivity. It is this approach that will be utilized in this research for detecting cancer cells.

Figure 8 (a) is the AFM image of a single nanotube coated with primary antibody in PBS after incubation for 2 hours followed by rinsing in distilled water and drying. Preliminary work on the conductance of nanotubes and nanotubes functionalized with antibodies has been studied recently by using atomic force microscope tips that is attached to the tips of a semiconductor prober attached to a parameter analyzer. The AFM tips that were used has a tip radius of 10 nm and tip height of 17 nm. The tips are attached by bonding the tungsten probe tip directly to the base of the AFM tip. Following bonding, 10 nm of gold is evaporated making the tips suitable for electrical measurements. The tips are mounted onto the probe station attached to a semiconductor parameter analyzer.

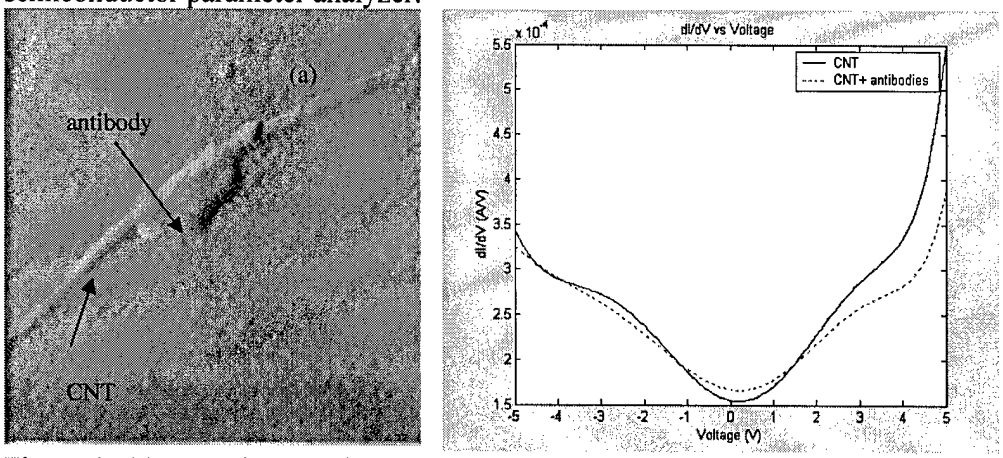

Figure 8: (a) AFM image of primary antibody functionalized on SWNT, the scan size is 1.25 x 1.25 μm. (b) Change in I-V as a function of the applied voltage between -5 to +5 V for CNT and

antibody functionalized CNT in PBS after an incubation period of 2 hours followed by rinsing in DI water and drying in air.

Figure 8(b) is the data graph of the differential conductance vs voltage between -5V to +5 V measured at room temperature for nanotubes from PBS with out the antibody and the nanotube functionalized with primary antibody in phosphate buffer solution after incubation period of 2 hours. The conductance was measured after rinsing the samples in water and drying them in air. The dip in the conductance plot shows the conductance gap. It can be seen that as the nanotubes are functionalized with the primary antibodies, it results in an increase in the conductance gap due to the broadening of the curve. The nanotubes that were used for this study were semiconducting in nature. This is the first study to show that the quantum electronic properties can be altered by antibody functionalization and is a fundamental step towards realizing sophisticated biological sensor systems in the future. By using metallic nanotubes, the change in the differential conductance can be increased to many orders of magnitude. When antibodies are functionalized on metallic nanotubes, it could render the nanotubes from metallic to semiconducting that can produce a large change in the conductance. It is expected that when the antibodies docks with the HER receptor, it will increase the conductivity that is expected in the course of this research, which also forms the basis of our sensor.

II. E. Detection and Killing of Cancer Cells in Culture:

This is an ongoing study and we have just started to characterize the interaction of nanotubes coated with antibodies in cell experiments. The functionalization procedure of antibodies on carbon nanotubes took most of the time allotted for this project. The reason is because of lack of any standard functionalization procedure and each and every step had to be characterized and quantified separately. However, the positive aspect of this research is the functionalization of primary antibodies on carbon nanotubes is very repeatable. We have atleast been able to functionalize six different samples with the same value of WCC anywhere between 88-90%, which shows that nanotubes could be used as delivery vehicles. Further, the conductance measurements are repeatable which shows that we are clearly in the correct path and our hypothesis of the biosensor using quantum electronic properties is correct. The present task at work should take about 3-6 months. With a no cost extension, the PI will be able to evaluate the interaction of antibody coated nanotubes in cell cultures and measure the WCC for both normal and cancer cells. Then cell killing can be achieved by using superoxide bismutase that is coated on the nanotubes similar to antibodies or by shining infra-red light on the nanotube that will heat the nanotubes to high temperature. This has never been done in the past and is an effective way of killing cancer cells. Further, by coating nanotubes with Tarceva and 2C4, one can promote anti-cancer activity in cell cultures which will also be studied.

III. Key Research Accomplishments:

The following are the key research accomplishments that have emanated through this research:

- This is the first time to show the effective separation of carbon nanotubes and their labeling to be viewed in confocal microscopy. The smallest nanotubes observed were about 10 nm.
- The use of surfactant and labeling using $DiOC_6$ has shown to increase the contrast in confocal imaging which shows the utility of carbon nanotubes for high contrast imaging with techniques such as MRI
- This is the first study to show antibody functionalization of carbon nanotubes for direct applications in breast cancer research. Previous research had pointed that antibodies cannot be functionalized on nanotubes due to their size and geometry. We have proved that it can be done through effective separation of the nanotubes using surfactants and labeling.
- Specific functionalization has been achieved by using di-amino PEG molecules to the nanotube surfaces that can attach to the primary antibody.
- This is the first study to show the differences in quantum conductance of nanotubes and nanotubes coated with antibodies. Coating nanotubes with antibodies shifts their energy gap and the associated conductance which can be measured using nano-probes, which is also the basis of the biosensor that is being developed for cancer detection applications.

IV. Reportable Outcomes:

The following are the reportable outcomes of this award:

- Several manuscripts have been lined up that illustrates the utility of nanotubes for breast cancer research
- A presentation has been scheduled at the IEEE Sensors conference in Vienna in Biosensors. This is a very prestigious conference with an acceptance rate of 55% and top 10% are allowed presentations. The PI is one of the top 10 presenters at the conference.
- The PI has been invited to be on the editorial board of the Journal Nanobiotechnology that caters directly to the medical applications of nanotechnology. This wouldn't have been possible with out the dissemination of the current results.
- A student Ranjani Sirdeshmukh has obtained her Masters degree due to this research and her thesis is "Antibody Functionalization of Carbon Nanotubes".
- A patent has been applied on the antibody functionalization of carbon nanotube and the biosensors based on this award.
- An NIH grant (R22/R33) has been applied from the results generated through this award for further study in animals and human subjects.
- Collaborations with medical schools such as Thomas Jefferson University has been strengthened due to this award.
- Collaborations with Oakridge National Laboratory has been established due to this award.

V. Conclusions:

In this research, a new technique of using carbon nanotubes for detection, diagnosis and killing of cancer cells have been investigated. The most important implications of this research is to be able to demonstrate the functionalization of antibodies on carbon nanotubes. The high degree of functionalization is achieved due to the well separation and labeling of carbon nanotubes using surfactant and flourophore solutions. This first study demonstrates the utility of nanotubes as an epitope for delivering antibodies to cells and tissues for cancer detection. Further, tumor suppressing agents such as Tarceva and 2C4, which are monoclonal antibodies can be functionalized on carbon nanotubes and selectively delivered for suppressing tumor and promoting anti-cancer activity. The continuation of this grant will allow us to study the effect of cell interactions on antibodies coated on nanotubes, measure their electronic properties and also investigate cell killing using nanotubes by heating the nanotubes using visible-Infra-red light. The potential applications of this study are enormous as this is the first time to conclusively show that the electronic properties of nanotubes can be altered by biomolecular functionalization. It will help in the development of a cancer diagnostic system that can detect cancer cells by investigating the changes in the surface electronic properties of carbon nanotubes. Further, the optical fluorescence of nanotubes can be utilized to image cells and tissues at the sub-100 nm limits using conventional microscopic techniques. This study also shows the utility of nanotubes as contrast agents for high resolution sub-micron to sub-100 nm MRI imaging which is not possible using present day technology. The potential applications of nanotubes for cancer research are therefore enormous.

VI. References

[1] M. F. Islam, E. Rojas, D. M. Bergey, A. T. Johnson, and A. G. Yodh, "High Weight Fraction Surfactant Solubilization of Single-Wall Carbon Nanotubes in Water," *NanoLetters*, pp. 269-273, 2003.

[2] O. Matarredona, H. Rhoads, Z. Li, J. H. Harwell, L. Balzano, and D. E. Resasco, "Dispersion of Single-walled carbon nanotubes in aqueous solutions of anionic surfactant NaDDBS," *Journal of Physical Chemistry B*, vol. 107, pp. 13357-13367, 2003.

[3] R. Prakash, S. Washburn, R. Superfine, R. E. Cheney, and M. R. Falvo, "Visualization of individual carbon nanotubes with flourescence microscopy using conventional flourophores," *Applied Physics Letters*, vol. 83, pp. 1219-1221, 2003.

[4] P. C. Cheng, T. H. Lin, W. L. Wu, and J. L. Wu, *Multidimensional Microscopy*, Springer Verlag, New York, 1994.

[5] E. M. M. Manders, F. J. Verbeek, and J. A. Aten, "Measurement of Colocalization of Objects in Dual Color Confocal Images," *Journal of Microscopy*, pp. 375-382, 1993.

[6] K. T. R. Sirdeshmukh, B. Panchapakesan, "Antibdoy Functionalization of Carbon Nanotubes," *Materials Research Society Spring Conference*, 2004.

[7] B. C. Braden, "X-ray Crystal Structure of an anti-Buckministerfullerene Antibody Fab Fragment: Biomolecular Recognition of C60," *Proceedings of the National Academy of Sciences*, pp. 12193-12197, 2000.

[8] B. C. B F Erlanger, M Zhu, L Brus, "Binding of an Anti-Fullerene IgG Monoclonal Antibody to Single Wall Carbon Nanotubes," *NanoLetters*, pp. 465-467, 2001.

[9] N. W. S. K. M Shim, R J Chen, Y Li, H Dai,, "Functionalization of Carbon Nanotubes for Biocompatibility and Biomolecular Recognition," *NanoLetters*, pp. 285-288, 2002.

[10] K. Sattler, "The energy gap of clusters nanopraticles and quantum dots", Hand book of thin film materials," *edited by H.S. Nalwa, Academic Press*, 2002.

VII. Appendices

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3. R.E. Cavicchi, R.M. Walton, J.Allen, M. Aquino-Class, and **B.Panchapakesan**, Spin-on nanoparticle tin oxide for microhotplate gas sensors. **Sensors & Actuators B** 77 (1-2), 2001, pp. 145-154.

4. S.Semancik, R.E. Cavicchi, C. Wheeler, R.M. Walton, J.E. Tiffany, J.S. Suehle, **B.Panchapakesan**, and D.DeVoe. Microhotplate platforms for chemical sensor research. **Sensors & Actuators B** 77 (1-2) 2001, pp. 579-591.

5. **B. Panchapakesan**, D. DeVoe, R.E. Cavicchi, and S. Semancik. Control of Tin Oxide Nanostructures on Arrays of Microhotplates. Proceedings of the 199th **Electrochemical Society** Meeting, Washington DC, March 2001.

6. **B.Panchapakesan**, D.DeVoe, R.Walton, R.Cavicchi, and S.Semancik. Micromachined Array Studies of Tin Oxide Films:Nucleation, Structure, and Gas Sensing Characteristics. Proceedings of spring **MRS** meeting, Vol.576, San Fransisco, April, 1999.

7. R.E.Cavicchi, S.Semancik, R.Walton, **B.Panchapakesan**, D.DeVoe, M. Aquino-Class, J.Allen, and J.S.Suehle, Micro-hotplate Gas Sensor Arrays. Proceedings of **SPIE**, v 3857, 1999.

8. R.Walton, C.Kendrick, R.Cavicchi, S.Semancik, **B.Panchapakesan**, and D.DeVoe. Processing methods for selected area film deposition on microsensor platforms using thermal and potential control. **Transducers '99**, The 10th International Conference on Solid-State Sensors and Actuators, Digest of Technical Papers, Volume 1, pp.676-679, Sendai, Japan, 1999.

9. J.Morillo, **B.Panchapakesan**, Q.Su, D.Novotny and M.Wuttig. Micromachined torsional resonator for magnetic anisotropy measurement. **Review of Scientific Instruments**, v 69, no.11, pp.3908-3912, November, 1998.

10. **B.Panchapakesan**, P.F. Mead, and Z. Fathi., "Variable Frequency Microwave Technology: A New Approach to Curing," **Advanced Packaging Magazine**, published September 1997.

11. Q.Y. Chen, P.F. Mead, **B. Panchapakesan**, and D. Haislett, "Physics of Failure Study of Optoelectronic Laser Transmitter Module", ASME Annual Conference, Atlanta, GA., November 1996.

Antibody Functionalization of Carbon Nanotubes for Breast Cancer Applications

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Abstract
Carbon nanotubes (CNTs) are remarkable solid state nanomaterials due to their unique electrical and mechanical properties. The electronic properties of nanotubes combined with biological molecules such as proteins could make miniature devices for biological sensing applications. In this paper, the interaction of single wall carbon nanotubes (SWNTs) with antibodies are presented. This approach is used towards developing a biosensor for breast cancer detection, by functionalizing the CNTs with antibodies that are specific to cell surface receptors of breast cancer cells. The degree of binding of antibodies on CNTs was found to be 67-88% by confocal microscopy. The key to achieve such high degree of functionalization is due to the separation of CNTs using surfactants that leads to a high surface area to volume ratio and higher number of active sites for charge transfer that enhances binding. Further, van der Waals forces and the charges on the antibodies and SWNTs are manipulated to achieve specificity of binding.

Keywords
Antibody Functionalization, Breast Cancer, Carbon Nanotube, Drug Delivery.

INTRODUCTION
Carbon nanotubes (CNTs) are novel synthetic materials with unique electronic [1] and superior mechanical properties [2]. They can be either metallic or semiconducting depending upon their diameter and chirality [3], thereby offering possibilities to form metal-semiconductor and semiconductor-semiconductor junctions, useful in electronic and sensor devices. Carbon nanotubes have found applications as building blocks for nanodevices such as probes [4], electron-field emission sources [5] and chemical sensors [6]. In addition, potential biological devices, which are fabricated by integrating nanotubes with organic molecules [7-9] will enable new research fields and applications such as in situ modification of living cells or their physiological activities [10].

The research of carbon nanotube functionalization has been intensified due to their great potential for biomedical and biotechnological applications. Organic modification of carbon nanotubes generates multiple sites for the attachment of bioactive molecules, and the modified nanotube could be used as a biosensor or a novel delivery system. Therefore, the understanding the interaction of carbon nanotubes with biological systems is essential for the realization of bio-hybrid systems. In very recent studies, nanotubes have been functionalized to study their biocompatibility and protein recognition capability. Davis et al. [11] reported the immobilization of proteins and enzymes in carbon nanotubes. They claimed that small proteins and enzymes can be readily placed within the interior cavity of opened nanotubes without any drastic conformational change. Chen et al. [12] demonstrated protein binding to SWNTs through a noncovalent sidewall functionalization scheme. A variety of smaller proteins, such as streptavidin and ferritin, have been immobilized on SWNTs that were functionalized by 1-pyrene butanoic acid succinimidyl ester. The pyrenel group irreversibly adsorbs onto the hydrophobic surfaces of SWNTs through π-π interaction, and the succinimidyl ester group reacts with amine groups on lysine residues of proteins to form amide bonds. More recently, Shim et al. [13] investigated the adsorption behavior of the streptavidin/biotin system on SWNTs. They achieved specific binding of streptavidin onto SWNTs by co-functionalization of nanotubes with biotin and protein resistant polymers. In another study, carbon nanotubes were functionalized by bovine serum albumin (BSA) proteins via diimide–activated amidation [14]. They claimed that the vast majority of the protein species in the nanotube BSA conjugates remained bioactive.

Although functionalization of CNTs with smaller proteins (streptavidin ~60 kDa / biotin) has been studied recently[13], it is particularly important to study the interaction of larger antibodies (~150 kDa) on CNTs due to its direct application in biomedical nanotechnology for cancer detection. In order to develop carbon nanotube

based cancer diagnostic systems, the following studies need to be conducted: (i) separation of carbon nanotubes in aqueous solutions, (ii) functionalization of CNTs with antibodies that are specific to cancer cell surface receptors, (iii) assembling the antibody coated nanotubes with the cancer cells to detect over expressed cell surface receptors (HER2). We have functionalized CNTs with three different antibodies: (i) monoclonal mouse IgG, (ii) polyclonal rabbit anti-goat IgG, (iii) polyclonal goat-anti-mouse IgG. The monoclonal mouse antibody is sensitive to cell-surface receptors (HER2) on a breast cancer afflicted cell. It should be pointed that BT474 human breast cancer cells, overexpress HER2 (Her2+) and c-MYC oncogenes, but not the estrogen receptor (ER^-) or IGF1 receptor ($IGF1R^-$) with MCF7M human breast cancer cells, which express the estrogen receptor (ER^+) and overexpress cyclin D1 and c-MYC oncogenes and the IGF1 receptor ($IGF1R^+$) [15-17]. Therefore, nanotubes are used as probes for detecting and killing cancer cells by assembling specific anti-oncogene antibodies on nanotube surfaces for detecting over expressed cell surface receptors (HER2). The interaction between CNTs and antibodies is studied mainly using confocal microscopy. Confocal microscopy is a widely used tool for fluorescent imaging of biological objects. We have used confocal microscopy to view fluorescently tagged CNTs in a method similar to that of viewing biological materials such as antibodies to accurately analyze and quantify their interaction.

EXPERIMENTAL DETAILS

SWNT solution was prepared in DI water with a surfactant, sodium dodecyl benzene sulfonate (NADDBS) by agitating the solution for 24 hours. The NADDBS to CNT ratio is 20 by weight [18]. Following that the well-separated nanotubes were labeled with dihexyloxacarbocyanine iodide ($DiOC_6$), a dye that fluoresces at 488nm. The $DiOC_6$ was prepared at 2mg/ml in methanol and diluted in DI water just prior to use with the CNT solution. The dye and nanotube solution were mixed in a micro centrifuge tube at a 1:1 ratio and allowed to interact for 1 hour.

The antibody solutions were prepared in Phosphate Buffered Saline (PBS) (0.138M NaCl, 0.0027M KCl, pH 7.4) by diluting a 2mg/ml antibody solution with PBS to a ratio of 1:10 (antibody:PBS). Then, the antibody solutions were pre-labeled with Alexa 546 (Molecular Probes, Inc.), a dye that fluoresces at 543 nm. The dye labeled CNT solution and the pre-labeled antibody solution was mixed in a micro centrifuge tube and allowed to interact for up to 2 hours prior to confocal microscopy analysis. Centrifuging was done to the antibody solution, when necessary, to eliminate unnecessary fluorescence. A slightly different procedure was carried out to view the monoclonal mouse IgG antibodies by confocal microscopy, since the presence of bovine serum albumin (BSA) with the antibody interferes the labeling of the antibodies. Therefore, the unlabeled mouse antibody was first tagged with the fluorescently labeled polyclonal goat anti-mouse antibody which selectively binds to the monoclonal mouse antibody. Then, the monoclonal mouse and polyclonal goat anti-mouse antibody conjugate was introduced to the dye labeled CNT solution. Besides confocal microscopy, scanning electron microscopy (SEM), and atomic force microscopy (AFM) were used for analysis.

RESULTS & DISCUSSIONS

Figure 1 is SEM images of CNTs showing the effectiveness of CNT separation using the surfactant, NaDDBS. This is clearly one of the most important steps to investigate functionalization of antibodies on nanotube surfaces due to achieving very high surface area to volume ratio. Further, water dispersion of CNTs has significant implications in biochemistry and biomedical engineering, in which organic solvents cannot be used due to their incompatibilities with living cells and organisms. The NaDDBS has the ability to break up the CNT bundles into individual nanotubes without forming chemical bonds. It has been reported [19] that during the adsorption of the anionic surfactant NaDDBS on

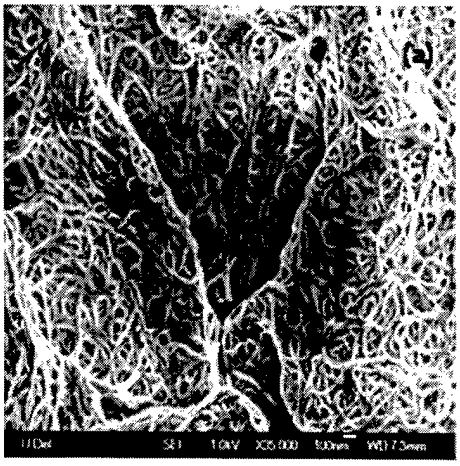
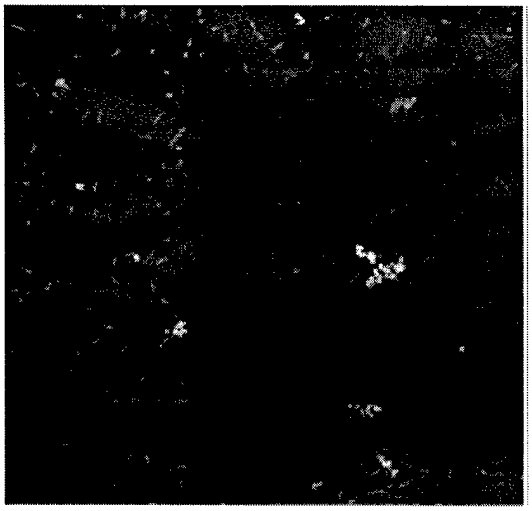


Figure 1. (a) SEM image of CNTs in water without the surfactant (non-separated); (b) SEM image of the separated CNTs in water with the surfactant at a ratio of 1:20 (CNT:Surfactant).

SWNTs, Columbic forces do not play a central role, but are overcome by the hydrophobic interactions between the surfactant and the nanotube walls. The well-separated nanotubes were then labeled $DiOC_6$, due to the fact that CNTs are too small to observe without the aid of fluorescent tags. Although it may be possible to image the larger CNT bundles with phase or differential interference contrast imaging with the help of video enhancement, fluorescence labeling allows individual small CNTs and attached molecules to be imaged with confocal microscopy. Noncovalent attachment of $DiOC_6$ to the CNTs allows imaging the entire CNTs without altering sp^2 bonded graphene sidewall. It was reported [20] that the noncovalent attachment of the dye to the CNTs could occur through hydrophobic interactions. It is believed that the binding was mediated through the hydrocarbon chains. Long hydrophobic hydrocarbon chains of the $DiOC_6$ would allow stable binding with the CNTs. Furthermore, the separation of the CNTs significantly enhanced the labeling of the CNT efficiency by $DiOC_6$.



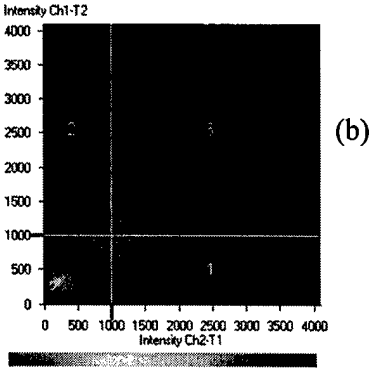


Figure 2. (a) Confocal microscopy image of a green-dye-labeled CNTs and subsequently coated with the red-dye-conjugated antibodies The green dye (labeling CNTs) in the inner rectangular region is bleached to verify the selective attachment of the antibodies to the CNTs (i.e. CNTs appear in red color due to the antibody binding); (b) Scattergram to measure the co-localization between antibodies and CNTs via calculation of WCC.

Having successfully achieved the separation and labeling of carbon nanotubes, the antibody functionalization of the CNTs has been carried out. Figure 2a is an overlap confocal image of CNTs (green) and antibodies (red). The green dye in the inner rectangular region is bleached out to verify the attachment of the antibodies to the CNTs. In order to measure the degree of binding, weighted co-localization coefficients (WCC) for both the CNTs and antibodies were calculated. WCC can be defined as the ratio of the intensity of co-localized area of a particular channel (color) to the intensity of total area above threshold intensity of that channel (color). It was reported that the co-localization coefficients can provide quantitative information in dual-color images [21]. Figure 2b is a scattergram which provides quantitative information about the image. The WCCs were found to be over 85% for both the CNTs and antibodies. The key to such high WCC is due to the separation of carbon nanotubes using a surfactant solution. When nanotubes are separated using a surfactant, it yields high surface area that enables attachment of antibodies on the nanotube surface without affecting the side wall, thereby yielding high WCC. Figure 3 is an AFM image showing the attachment of the antibodies to a single nanotube. This indicates that surface tension and

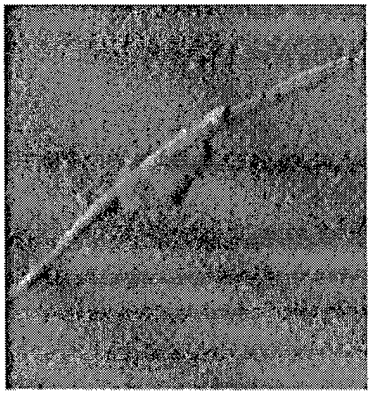


Figure 3. AFM image of the same sample (Fig.2a) showing the attachment of the antibodies to a single CNT. The scan size is 1.25 x 1.25μm.

van der Waals forces can be utilized for binding antibodies to CNTs for biomedical applications.

CONCLUSIONS

We have successfully demonstrated antibody functionalization of carbon nanotubes. Initially, the CNT bundles were separated to individual nanotubes in aqueous solutions. Following that the individual CNTs were functionalized with antibodies. In fact, very high degree of binding and co-localization has been observed by confocal microscopy. This suggests that nanotubes can be used as probes for detecting cancer cells by assembling specific anti-oncogene antibodies on nanotube surfaces for detecting over expressed cell surface receptors (HER2).

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REFERENCES

[1] M. Bockrath, D.H. Cobden, P.L. McEuen, N.G. Chopra, A. Zettl, A. Thess, R.E. Smalley, "Single-Electron Transport in Ropes of Carbon Nanotubes", Science, vol. 275, pp. 1922-1925, (1997).

[2] R.S. Ruoff, D.C. Lorents, "Mechanical and Thermal Properties of Carbon Nanotubes", Carbon, vol. 33, pp. 925-930, (1995).

[3] P. Avouris, "Carbon Nanotube Electronics", Chem. Phys., vol. 281, pp. 429, (2002).

[4] H. Dai, J. H. Hafner, A. G. Rinzler, D. T. Colbert, R. E. Smalley, " Nanotubes as Nanoprobes in Scanning Probe Microscopy", Nature, vol. 384, pp. 147, (1996).

[5] W. A. De Heer, A. Chatelain, D. Ugarte, "A Carbon Nanotube Field-Emission Electron Source", Science, vol. 270, pp. 1179, (1995).

[6] J. Kong, N. R. Franklin, C. Zhou, M. G. Chapline, S. Peng, K. Cho, H. Dai, " Nanotube Molecular Wires as Chemical Sensors", Science, vol. 287, pp. 622, (2000).

[7] S. S. Wong, E. Joselevich, A. Woolley, C. L. Cheung, C. M. Lieber, " Covalently Functionalized Nanotubes as Nanometer-Sized Probes in Chemistry and Biology", Nature, vol. 394, pp. 52, (1998).

[8] A. Koshino, M. Yudasaka, M. Zhang, S. Iijima, "A Simple Way to Chemically React Single Wall Carbon Nanotubes with Organic Materials using Ultrasonication', Nano. Lett., vol. 1, pp. 361, (2001).

[9] V. Georgakilas, K. Kordatos, M. Prato, D. M. Guldi, M. Holzinger, A. Hirsch, "Organic Functionalization of Carbon Nanotubes", J. Am. Chem., vol. 124, pp. 760, (2002).

[10] K. Otobe, H. Nakao, H. Hayashi, F. Nihey, M. Yudasaka, S. Iijima, "Fluorescence Visualization of Carbon Nanotubes by Modification with Silicon-Based Polymer", Nano. Lett., vol. 2, pp. 1157, (2002).

[11] J.J. Davis et al, "The Immobilization of Proteins in Carbon Nanotubes", Inorg. Chem. Acta, vol. 272, pp. 261-266, (1998).

[12] R.J. Chen, Y. Zhang, D. Wang, and H. Dai, "Noncovalent Sidewall Functionalization of Single-Walled Carbon Nanotubes for Protein Immobilization", J. Am. Chem. Soc., vol. 123, pp. 3838-3839, (2001).

[13] M. Shim, N. W. S. Kam, R. J. Chen, Y. Li, H. Dai, "Functionalization of Carbon Nanotubes for Biocompatibility and Biomolecular Recognition", Nano Lett., vol. 2, pp. 285-288, (2002).

[14] W. Huang et al., "Attaching Proteins to Carbon Nanotubes via Diimide-Activated Amidation" Nano Lett., vol 2, pp. 311-314, (2002).

[15] D. F. Hayes, T. M. Walker et al., "Monitoring expression of HER-2 on circulating epithelial cells in patients with advanced breast cancer", Int. J. Onco., vol 21 (5), pp. 1111-1117, (2002).

[16] M. A., Guvakova, E. Surmacz, "Overexpressed IGF-I receptors reduce estrogen growth requirements, enhance survival, and promote E-cadherin-mediated cell-cell adhesion in human breast cancer cells", Exp. Cell. Res., vol. 231 (1), pp. 149-162, (1997).

[17] X. Le Roy, C. Escot et al., "Decrease of c-erbB-2 and c-myc RNA levels in tamoxifen-treated breast cancer", Oncogene, vol. 6 (3), pp. 431-437, (1991).

[18] M. F. Islam, E. Rojas, D. M. Bergey, A. T. Johnson, A. G. Yodh, "High Weight Fraction Surfactant Solubilization of Single-Wall Carbon Nanotubes in Water", Nano Lett., vol. 3, pp. 269-273, (2003).

[19] O. Matarredona et al., " Dispersion of Single-Walled carbon Nanotubes in Aqueous Solutions of the Anionic Surfactant NADDBS", J. Phys. Chem. B, vol. 107, pp. 13357, (2003).

[20] R. Prakash, S. Washburn, R. Superfine, R. E. Cheney, M. R. Falvo, "Visualization of Individual Carbon Nanotubes with Fluorescence Microscopy Using Conventional Fluorophores", Appl. Phys. Lett., vol. 83, pp. 1219-1221, (2003).

[21] E. M. M. Manders, F. J. Verbeek, J. A. Aten, "Measurement of Colocalization of Objects in Dual Color Confocal Images", J. Microscopy, vol. 169, pp.375-382, (1993)